Review of the Draft NTP Acrylamide Technical Report (TR-575)

Prepared by Dr. Joseph K. Haseman at the request of NAPPA
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During my 33 years at the NIEHS, I was the biostatistician included in the core group of NTP scientists given the responsibility of interpreting experimental results and preparing NTP Technical Reports that summarized NTP's rodent cancer bioassays. Because of this experience, NAPPA asked me to review the Draft NTP Acrylamide Technical Report (TR-575) prepared by the NCTR and give an opinion regarding the appropriate level of evidence of carcinogenic activity in this two year drinking water study.

In my opinion, the multi-site carcinogenicity seen in all four sex-species groups justifies the "clear evidence" calls. However, I do not agree with all the specific tumors that are part of the calls and/or are listed in the Abstract Summary Table. Specifically, I recommend that the following tumors be deleted from the calls: (i) pancreatic islet cell adenoma in male rats; (ii) clitoral gland carcinoma in female rats; and (iii) skin squamous cell tumors in female mice. The marginal malignant schwannoma effect in female rats should also be appropriately categorized as an equivocal finding in the Abstract Summary Table to be consistent with the call made by the NTP for this tumor.

In addition, the levels of evidence calls for mammary gland fibroadenoma in female rats and mesenchymal skin tumors in female mice should also be reconsidered and possibly downgraded in light of relevant historical control data that weaken the statistical (and biological) significance of these findings. The justification for these recommendations is given below, along with discussion of other issues.

I. NCTR historical control data

Perhaps the most important issue deals with the historical control data. Historical control data are potentially important in the interpretation of certain tumors, but there are problems with the historical control database for F344/N rats and B6C3F1 mice that the NCTR relied on in the acrylamide TR. These problems include (i) most of the studies are 20-23 years old; (ii) none of the studies for rats (and only one for mice) are drinking water studies; (iii) the recent NTP drinking water studies are ignored; and (iv) the historical control tumor rates reported by the NCTR in the acrylamide TR are often wrong.

Old studies. Most of the NCTR historical control data for F344/N rats and B6C3F1 mice are from studies that are 20-23 years old (1988-1991), and from studies that were never published as NTP Technical Reports. Throughout its history, the NTP has consistently used a "time window" of approximately 5-10 years for historical control data, so that the studies in the historical control database are reasonably concurrent with the study in question. This is important, because study protocols and background tumor rates change over time. I feel quite strongly that 23 year old historical control data should not be used to interpret the results of a rodent carcinogenicity study. Moreover, these old studies were all diet studies, whereas the acrylamide study is a drinking water study. This is also

important, since certain tumors in the NCTR database differ significantly in incidence between drinking water and diet controls, as discussed below.

I strongly recommend that the historical control database used in the acrylamide TR eliminate all of the 20-23 year old studies. For rats, that would leave only three relatively recent studies: Fumonisin B1, Malachite Green (females only), and Leucomalachite Green. Unfortunately, none of these studies are drinking water studies. A similar approach should be used for mice, which would include one relatively recent drinking water study among the historical controls in the NCTR database (Urethane and Ethanol).

NTP Drinking Water Studies. In addition to the NCTR database, there are many NTP studies using F344/N rats and B6C3F1 mice that provide relevant historical control data. These include a number of recent drinking water studies. In my opinion, these recent NTP drinking water studies are far more relevant than the 23 year old unpublished NCTR dietary controls for interpreting the results of the acrylamide drinking water study. Thus, I recommend that the NCTR replace the older dietary controls with the more recent NTP drinking water controls.

Inaccurate Tumor Rates: Pancreatic islet cell tumors in male rats. One problem with the NCTR historical control database is that the NCTR is often unable to find and/or to accurately report the correct tumor rates. Perhaps the most obvious example of this is pancreatic islet cell adenoma in male rats. In Table A3d, the NCTR reports the historical control incidence to be zero among 530 control animals from six previous studies, but 9/47 (19.1%) in a seventh study (Fumonisin B1). This difference among control groups is highly significant ($p<0.000001$), suggesting that something was wrong. And so it was.

Since the data from the 20-23 year old studies were unavailable, I checked the only recent study (Leucomalachite Green) that purportedly had a zero pancreatic islet cell adenoma incidence, and I found that the control incidence of pancreatic islet cell adenoma in male rats was actually 5/48, not 0/48, as reported in Table A3d.

What went wrong? The NCTR apparently confused pancreatic ACINAR cell tumors and pancreatic ISLET cell adenoma (two totally different tumor types) and overlooked the fact that pancreatic islet cell tumors were listed under “islet”, not under “pancreas” in the appendix table in the Leucomalachite Green study that summarizes the incidence of neoplasms. As a result, the NCTR mis-reported the pancreatic islet cell tumor rates.

When I checked the other recent NCTR female rat study, I found a similar problem. NCTR reported that the control rate of pancreatic islet cell tumors in the Fumonisin B1 study was 9/47, but this was actually the rate for all pancreas tumors, including acinar cell tumors. If the acinar cell tumors are deleted, then the actual control rate of pancreatic islet cell adenoma/carcinoma in this study appears to be 7/47 (15%), consistent with the control rate in the Leucomalachite Green study (5/48).

Since these are only two relevant male rat historical control groups in the NCTR database (and neither are drinking water studies), the relatively large NTP historical control

database of drinking water studies should be included in the acrylamide TR. A comparison of historical rates of pancreatic islet cell adenoma/carcinoma in the various recent NTP drinking water control groups (see http://ntp.niehs.nih.gov/?objectid=92E6AAA5-F1F6-975E-71C88528A3E7B315 and http://ntp.niehs.nih.gov/?objectid=92E705C7-F1F6-975E-72D23026B1645EB9#10) is given below.

Acrylamide concurrent controls	Drinking water	1/46 (2%)
Acrylamide high dose	Drinking water	6/48 (13%)
NCTR database	Diet	12/95 (13%)
Recent NTP [NIH07 diet]	Drinking Water	38/324 (12%)
Recent NTP [NTP 2000 diet]	Drinking Water	15/148 (10%)

Note the consistency of tumor rates across the three historical control databases. This is very important. These data make it clear that the incidence of pancreatic islet cell adenoma seen in the top dose acrylamide group is essentially the incidence that would be expected based on the historical control data from both the NCTR and the NTP. It is the concurrent control rate (1/46, 2%) that is abnormally low, not the top dose rate that is abnormally high. Thus, I strongly recommend eliminating the pancreatic islet cell adenoma "increase" from the final call. The Discussion Section indicates that this particular tumor type was not a suspected target site in any case.

Incomplete Reporting of Tumor Rates: Clitoral gland neoplasms in female rats. The clitoral gland carcinoma call in female rats should also be eliminated. Clitoral gland adenoma/carcinoma is a continuum, and one should not consider carcinoma in isolation from adenoma. In the acrylamide study, the combined incidence of clitoral gland adenoma/carcinoma in the dosed groups is not even close to being statistically significant: (10/48, 13/48, 17/47, 11/48, 11/47; see Table B2).

The NTP has always given primary emphasis to combined tumor incidence in such situations Thus, for example, if there is a positive trend in carcinoma and a negative trend in adenoma (or vice versa) , so that the overall tumor rates are essentially identical, as is the case here, the overall call would be "no evidence."

It is interesting that when discussing the carcinogenic effects in female rats in the Abstract (page 9), the NCTR (correctly) emphasizes the combined tumor incidence (e.g., oral cavity papilloma/carcinoma, thyroid follicular gland adenoma/carcinoma, skin fibroma/fibrosarcoma/sarcoma), and yet in their discussion of clitoral gland tumors, they focus solely on the carcinomas. That is inconsistent and inappropriate, in my opinion.

I suspect that the "threshold" to distinguish between clitoral gland adenoma and carcinoma was inconsistently applied in the dosed and control groups. The controls reportedly had 9 adenomas and one carcinoma, while the high dose reportedly had 3 adenomas and 8 carcinomas. Thus, the overall clitoral gland tumor burden in these two groups (10 vs. 11) was essentially the same.

What about the historical control data? Table B3b reports that the historical control rate for clitoral gland carcinoma averages 8% and ranges as high as 40% (see Table B3b). Thus, the concurrent control rate (2%) is abnormally low, and the high dose rate of 17% falls well within the historical control range of 0-40%. Table B3b inexplicably fails to report the incidence of clitoral gland adenoma.

However, as noted earlier, for female rats, there are really only three relevant previous studies in the NCTR database (see Table B3b): Fumonisin B1, Leucomalachite Green, and Malachite Green. Table B3b incorrectly states that the clitoral gland tumor rates in the Leucomalachite Green study were "not reported", but I had no trouble finding them. The clitoral gland tumor rates in these three studies are given below

	Clitoral gland adenoma	Clitoral gland carcinoma
Fumonisin B1	10/41	1/41
Malachite Green	7/48	5/48
Leucomalachite Green	7/47	2/47

Thus, the overall control incidence of clitoral gland adenoma/carcinoma seen in these three NCTR control groups (32/136 or 24%) is essentially the same as the rate seen in the acrylamide high dose group (11/47, 23%).

What about the more extensive NTP database for drinking water studies? These control data are summarized below.

	Clitoral gland tumors		
	Adenoma	Carcinoma	Adenoma or carcinoma
Recent NTP [NIH07 diet]	7% (22/321)	4% (12/321	11% (34/321)
Recent NTP [NTP 2000 diet]	16% (24/150)	6% (9/150)	22% (33/150)

The NTP 2000 diet study controls are more recent than the NTP NIH07 study controls, and thus are more relevant for the interpretation of the acrylamide study. The overall control rate of clitoral gland tumors in this group (22%) is similar to the rate seen in the high dose acrylamide group (23%) and in the relevant NCTR controls (24%). In conclusion, I strongly recommend eliminating clitoral gland carcinoma from the call.

Drinking Water Controls and Mammary Gland Fibroadenoma. Another tumor whose call could be impacted by the inclusion of relevant historical control data is mammary gland fibroadenoma in female rats. The relevant tumor rates are given below.

Acrylamide concurrent controls	Drinking water	16/48 (33%)
Acrylamide high dose	Drinking water	31/48 (65%)
NCTR database	Diet	53/141 (38%)
Recent NTP [NIH07 diet]	Drinking Water	121/330 (37%)
Recent NTP [NTP 2000 diet]	Drinking Water	111/150 (74%)

The NTP 2000 diet controls are arguably the most relevant controls, since these studies are (i) drinking water studies; and (ii) most concurrent in time with the acrylamide study. However, using this reference group, there is obviously no effect of acrylamide on mammary gland fibroadenoma. In addition, the historical control rate of mammary gland fibroadenoma in female F344/N rats in the three recent NTP feeding studies using the NTP-2000 diet are 56% (28/50), 58% (29/50) and 60% (30/50), once again consistent with the rate of 65% seen in the top dose acrylamide group.

Since the rate of mammary gland fibroadenoma in the top dose acrylamide group is actually less that the average rate of this tumor seen in three recent NTP drinking water study control groups, a strong case could be made for downgrading this call from its current "clear evidence" status.

Mis-categorization of malignant schwannoma. Another problem involves malignant schwannoma in female rats. The historical control tumor rate for this tumor in NCTR studies is reported to be zero (0/636, see Table B3f), and yet the control rate in the acrylamide study is 2/48 (see Table B2). This difference is highly significant ($p<0.01$). Given this high concurrent control rate, the slightly increased incidence in the high dose group (4/48 vs. 2/48) becomes more problematic. The NCTR apparently recognized this and correctly (in my opinion) concluded (see page 11) that this marginal increase "may have been related to acrylamide exposure".

This language is very important, as the NTP uses such language to describe an "equivocal" or "uncertain" finding, as discussed below. However, the Abstract Summary Table lists no equivocal findings for female rats and includes this marginal increase in malignant schwannoma as a "neoplastic effect". This needs to be changed. This marginal increase in malignant schwannoma should be listed as an "equivocal finding" to be consistent with the NCTR's own interpretation of the data.

Non-effect Regarded as a Neoplastic Effect. The skin squamous cell carcinoma "effect" in female mice given in the Abstract Summary Table should be deleted. The incidence of squamous cell papilloma/carcinoma in female mice is not even close to being statistically significant (1/48, 0/46, 0/48, 1/45, 2/43; see Table D2), and these tumors are not mentioned anywhere in the Abstract (squamous cell tumors are not "mesenchymal tumors"). Thus, since the NCTR did not consider these tumors to be related to acrylamide, they should be deleted from the Abstract Summary table.

Differences between the NCTR Drinking Water and Dietary Control Databases. As noted previously, the NCTR historical control database had no rat drinking water studies, while the mice had only one. For mesenchymal skin tumors in female mice, the single drinking water study control has more than 10 times the incidence (4/48, 8.3%) as the diet controls (0.8%, 4/509). This significant difference between drinking water and dietary controls supports my recommendation to include the NTP drinking water studies in the TR's historical control database to have a more relevant set of historical control data. The historical control rate of mesenchymal skin tumors in female mice in the two

NTP historical control drinking water databases are 2.9% (10/340) and 5.3% (8/150), consistent with the rates in the NCTR drinking water study.

Since the incidences of mesenchymal skin tumors in dosed female mice (18% in the next to top dose group and 12% in the top dosed group) are not significant relative to the one NCTR drinking water study, it could be argued that this effect should be downgraded from its current "clear evidence" status.

Differences between Diets. It should be noted that the diet used in the NCTR acrylamide study (irradiated Purina 5LG6 meal food, available ad libitum; also referred to as NIH-31 IR) is different than the diets used in the NTP drinking water studies (NIH-07 or NTP 2000 diets). In fact, the NIH-31 IR diet appears to be different than the diet used in the recent NCTR dietary studies noted above, in that these other studies make no mention of the diet being irradiated, and report the diet simply as NIH-31.

Does the diet really make a difference in terms of tumor incidence? I would argue that for the majority of tumors, including most of those evaluated in this report, it does not (e.g., pancreatic islet cell tumors in male rats; skin mesenchymal tumors in female mice; other tumors I have examined but not discussed in this report, as they are unaffected by acrylamlide). These consistencies across databases reinforces certain of my conclusions (e.g., that the marginal increase in pancreatic islet cell adenoma seen in dosed male rats was unrelated to acrylamide).

Moreover, when differences across historical control databases do exist (e.g., mammary gland fibroadenoma in female rats), it is unclear whether these differences are related to differences in diet, temporal differences (older vs. more recent studies), type of control group (drinking water vs. diet controls), or possibly even inherent differences among the F344 rats used at the NCTR and by the NTP. This last hypothetical difference is not addressed in this report, but I have found no published evidence to suggest that the control tumor rates among NCTR F344/N rats differ significantly from the corresponding control rates seen in comparable NTP F344/N rats.

Thus, in my opinion, the historical control tumor data from the NTP drinking water studies should definitively be included as part of the interpretative process. It is far more important to have reasonably contemporary controls that are from the same type of study (i.e., drinking water studies) rather than insisting that the diets be identical.

Other Misreported Tumor rates. The NCTR incorrectly reports (Table A3b) that the control incidence of mesothelioma (all sites) in male rats was "not reported" in the Leucomalachite Green study. It was indeed reported and was 2/48. Table B3d incorrectly states that the incidence of oral cavity squamous cell tumors was "not reported" in the Malachite Green and Leucomalachite Green studies. In fact the incidences were reported and were 0/48 in both studies. Table B3g incorrectly reports the control incidence of hepatocellular adenoma in female rats in the malachite green study to be 0/48, whereas the true rate is 1/48. Table D3g incorrectly reports that the incidence of mescenchymal skin tumors was not reported in the leucomalachite green and

malachite green studies. In fact, the rates were reported and were 0/46 and 0/48 in the two studies respectively. These errors have little or no impact on the overall calls, but illustrate the difficulty the NCTR had when reporting their own historical control data.

Bottom line recommendation: the NCTR should (i) eliminate all of the 20-23 year old studies from the historical control database; (ii) recheck and correct when necessary the historical control tumor rates given in the appendices; (iii) include the recent NTP drinking water studies in the database to have a more relevant reference group for comparison; and (iv) re-interpret the experimental results in the light of the new and more appropriate historical control data.

II. Non-neoplastic Lesions

When one evaluates literally hundreds of non-neoplastic lesions, there are bound to be some false positives. Thus, I recommend that in the Abstract Summary Table (page 12) the NCTR limit the non-neoplastic effects to those that are (i) highly significant, (ii) biologically important; and (iii) identified as being significant non-neoplastic effects earlier in the Abstract. That is, the non-neoplastic lesions identified in the Conclusion on page 11 should correspond exactly with those listed in the Abstract Summary Table. The Abstract Summary Table lists 19 significant non-neoplastic effects, while the text in the Abstract Conclusion identifies only 13.

With this in mind, I recommend that the NCTR delete the following six marginally significant non-neoplastic effects from its Abstract Summary Table:

Male rats: Preputial gland duct ectasia; Female rats: Adrenal cortex hypertrophy and cytoplasmic vacuolization; ovary atrophy; bone marrow hyperplasia; Male Mice: Preputial gland inflammation (or add this to the list of non-neoplastic effects on page 11); and Female mice: None

III. Tumor Combinations

In this study, The NCTR combined certain tumors for statistical analysis that are of different cell types and thus should be evaluated separately. These inappropriate tumor combinations include: (i) “all morphologies” of the skin (i.e., fibroma, sarcoma, keratoacanthoma, basal or squamous cell papilloma/carcinoma, hemangiosarcoma, liposarcoma, myxosarcoma, fibrous histiocytoma, neurofibrosarcoma), (ii) thyroid c-cell and follicular cell tumors; and (iii) forestomach squamous cell papilloma, squamous cell carcinoma and sarcoma. These inappropriate tumor combinations should all be deleted from the TR.

IV. Minor typos

In the middle of page 10, “squamous cell adenoma” should be “squamous cell papilloma”. In the last paragraph of page 10, mammary gland is not identified as the

organ showing adenocarcinoma/adenoacanthoma, and adenoacanthoma is disucussed twice in the same paragraph in different places.

V. Final Calls

It is important to understand the NTP's wording of the final conclusion when there is a "clear evidence" call. First, the tumors are listed that provide clear evidence of carcinogenic activity. NTP then gives the lesser "positives" that individually would be called "some evidence" of carcinogenic activity, but since the NTP does not use multiple categories for the same sex-species group, the language that it uses in such cases is "were considered to be related". Finally, for those tumors that individually would have been labeled "equivocal evidence of carcinogenic activity", the NTP uses the language "may have been related".

For example, for female rats in the acrylamide study, the mammary gland, oral cavity, mesenchymal tumors of the skin, and thyroid follicular cell tumors were collectively regarded as providing "clear evidence" of carcinogenic activity. In addition, the clitoral gland and liver tumors were considered to provide (in effect) "some evidence" of carcinogenic activity, while the malignant schwannoma of the heart was considered to be an equivocal effect. Now for the calls:

I agree with the clear evidence calls, but recommend the following changes:

(a) Male rats: Eliminate pancreatic islet cell adenoma from the call.

(b) Female rats: Eliminate the clitoral gland carcinoma call; properly categorize malignant schwannoma of the heart as an "Equivocal Finding" in the Abstract Summary Table.

(c) Male mice: No change.

(d) Female mice: Eliminate squamous cell carcinoma of the skin from the Abstract Summary Table.

It is important to emphasize that these recommended changes are NOT dependent upon whether or not the NTP historical control data are considered appropriate. While this is an important issue, and I feel that these data are appropriate, the recommended changes in the conclusions are based primarily on the data from the acrylamide study itself and the (corrected) NCTR historical control data. However, the NTP historical control data do provide additional support to these recommended changes.

Finally, the levels of evidence calls for mammary gland fibroadenoma in female rats and mesenchymal skin tumors in female mice should also be reconsidered and possibly downgraded, because relevant historical control data weaken the statistical (and biological) significance of these findings.

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